Chapter Four

How do soil nutrients affect within-plant patterns of herbivory in seedlings of *Eucalyptus nitens*?

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Abstract

This study assessed how the palatability of leaves of different age classes (young, intermediate and older) of *Eucalyptus nitens* seedlings varied with plant nutrient status, based on captive feeding trials with two mammalian herbivores, Red-bellied Pademelons (*Thylogale billardierii*), and Common Brushtail Possums (*Trichosurus vulpecula*). Seedlings were grown under three nutrient treatments (low, medium and high) and we determined how palatability was related to chemical and physical characteristics of the leaves. Pademelons ate more older leaves than young and intermediate leaves for all treatments. This pattern was best explained by sideroxylonals (formylated pholoroglucinol compounds known to deter herbivory by other marsupials), and/or essential oil compounds that were in lower concentrations in older leaves. In the low nutrient treatment, possums also ate more of the older leaves. However, in the medium and high nutrient treatments, possums ate more intermediate leaves than older leaves and showed a behavioural preference for young leaves (consuming younger leaves first) over intermediate and older leaves, in spite of high levels of sideroxylonals and essential oils. The young leaves did, however, have the highest nitrogen concentration of the leaf age classes. Thus, either sideroxylonals and essential oils provided little or no deterrent to possums, or the deterrent was outweighed by other factors such as high nitrogen. This study indicates that mammalian herbivores show different levels of relative use and damage to leaf age classes at varying levels of plant nutrient status and, therefore, their impact on plant fitness may vary with environment.

Introduction

The impact of herbivores on the growth and survival of plants depends on the severity of damage to the plant, and which plant parts are eaten. This, in turn, is affected by the physical and chemical characteristics of the plant. Chemical characteristics of foliage are well known to influence herbivory (eg. O'Reilly-Wapstra et al. 2004, Wiggins 2006), through toxicity or reduced nutritive value of the plant to the animal. Effects of physical characteristics on herbivory have been less well studied, but may be important (Sanson et al. 2001). This is well demonstrated for insects (Lowman and Box 1983, Ohmart and Edwards 1991, Steinbauer et al. 1998) but is less well known for mammalian herbivores.

Chemical and physical characteristics of plants that affect palatability to herbivores are often affected by characteristics of the environment, such as light and nutrient availability. Several studies have investigated environmental effects on characteristics within plants. For example, Close et al. (2005) demonstrated that varying nutrient availability to *Eucalyptus nitens* seedlings at the whole plant level can alter chemical characteristics of leaves belonging to different age classes. Furthermore, previous studies have shown that different nutrient regimes can alter the chemistry and palatability of *E. nitens* at the whole plant level (Close et al. 2003, McArthur et al. 2003). The impacts of differing chemical and physical characteristics within *E. nitens* has yet to be investigated in relation to herbivory.

Recent attention in community ecology has focused on how removal of a preferred species of seedling can affect plant community composition (Hanley 1998). More subtly, selective herbivory within plants may also affect plant communities. For example, in tree seedlings with apical dominance, repeated browsing of the apical bud can change the final form from a tall single stem to a shorter, bushy form with multiple leaders (Volker and Orme 1988, Bulinski and McArthur 1999). Such changes in growth form could influence community structure by altering canopy and ground cover, and the light regime in the community.

The main aim of our study was to determine how the palatability of leaf age classes (young, intermediate, older) within seedlings differed under altered nutrient conditions. We used two mammalian herbivores, a mixed feeder browser, the Red-bellied Pademelon (*Thylogale billardierii*) (Sanson 1989, Sprent and McArthur 2002), and a generalist folivore, the Common Brushtail Possum (*Trichosurus vulpecula*) (Freeland and Winter 1975), referred to hereafter as pademelons and possums. Both species consume eucalypt seedlings and respond to changes in plant chemistry at the whole plant level (Close et al. 2003, McArthur et al. 2003). We chose *E. nitens* because it shows large growth responses to fertiliser (Honeysett et al. 1992). We examined how changes in palatability were related to differences in the chemical and physical characteristics of leaf age classes. Primary constituents of nitrogen and fibre were also examined, along with several secondary metabolites that have previously been implicated in influencing intake by herbivores. These were essential oils, total phenolics and the formylated pholoroglucinol compounds sideroxylonals. Lambdon and Hassall (2005) suggest that herbivore selectivity for leaves of different ages within a plant is simple in comparison to selection of

leaves from different plants, as the chemical composition of leaves is usually less variable between leaves of different ages than between individual plants. In order to investigate this, and to make comparisons with other studies, chemical and physical characteristics were also quantified on the whole seedling basis.

Methods

Plant material, nutrient treatments, and leaf age classes

Seedlings of *Eucalyptus nitens* (Deane and Maiden) were raised from a multiple-family seedlot derived from 3-50 mothers and a large number of fathers. This genetic selection represented samples from the major provenances of *E. nitens*. A considerable amount of genetic variation has been demonstrated both between and within provenances of *E. nitens* (Pederick 1979), therefore the seedlings used in this study are likely to include a high proportion of the genetic diversity found within this species. Seedlings were grown in Lännen 81 (Lännen Plant Systems Oy, Säkylä, Finland) 85 cm^3 plugs at Forestry Tasmania's tree nursery, Perth, TAS, Australia (41º 34´ S, 147º 11´ E). For four months the potting mix was saturated with 1g.L^{-1} Aquasol (Hortico, Homebush, NSW, Australia) every 7 days (N:P:K 23:4:18). At the end of this time (early February, 2003), the seedlings were moved to an outdoor growing area at the Cooperative Research Centre for Forestry, Hobart, Tasmania, Australia, then fertilised weekly for one month with 1g.L^{-1} Peters Excel (Marysville, OH, USA) (N:P:K 20:2.2:6.6). On March 4th 2003 seedling trays were randomly allocated to one of three nutrient treatments: 1) low (no additional nutrient application), 2) medium (nutrients applied once weekly), 3) high (nutrients applied three times weekly). At each nutrient application each seedling received approximately 15 mg Peters Excel. Each treatment was replicated five times in a randomised block design oriented east west. Each of the 5 blocks consisted of 6 trays, each containing 81 seedlings. Treatments were applied for six months and seedlings were watered twice daily.

Leaves were classified into three leaf age classes: young, intermediate and older. Young leaves were the apical bud and the first leaf pair. The remaining leaves were divided evenly into intermediate and older leaves. If the number of remaining leaf pairs was odd, the extra leaf pair was allocated to the intermediate leaf age class.

Feeding trials comparing intakes

Feeding trials were performed with six pademelons (four males and two females, 5.4 - 7.5 kg body mass) and six possums (three males and three females, 3.6 - 4.2 kg body mass). Pademelons were part of a captive colony maintained in an animal enclosure at the School of Zoology, University of Tasmania, Hobart, Tasmania, Australia, while possums were wild caught. All animals were housed and maintained following McArthur *et al.* (2000).

We tested and compared the animals' relative use of specific leaf age classes within each nutrient treatment. When leaf classes were available *ad libitum*, we measured relative use in terms of leaf dry matter intake. When a leaf age class was not offered *ad libitum* and an accurate measure of intake under such conditions could therefore not be made, we used behavioural data to provide an

index of relative use, defined as the leaf age class predominately eaten first from the seedlings.

Each animal was offered seedlings from one nutrient treatment for a period of three nights. For the first two nights, animals were offered a basal diet equivalent to 100 % of their requirements to maintain body mass, and seedlings (15 for pademelons, 20 for possums) to familiarise the animals with the way that seedlings would be offered. On the third night, animals were offered basal diet equivalent to 50 % of their maintenance requirements, and many seedlings of the respective nutrient treatment (50 for pademelons, 100 for possums). Data from the third night within each period were used in statistical analyses. Seedlings of the three treatments were offered in a balanced cross-over design, to allow for a test of carryover effects of the previous treatment (Ratkowsky et al. 1993). Based on previous work, the number of seedlings was chosen to provide *ad libitum* quantities of all leaf age classes, although, as described in the results, this was not always successful. Between treatments, animals were offered basal diet equivalent to 120 % of their daily maintenance requirements for three days.

The height of each seedling and number of leaves in each age class (young, intermediate, older) was recorded. Seedlings offered to the animals were randomly selected from across blocks, with the same number of seedlings taken from each block. Seedlings were offered in wire racks and spaced to ensure that the animals had access to every seedling. Racks were placed in each cage in the afternoon (at ~ 1700 hours) and removed the following morning (at ~ 0800). Seedling height was re-measured and a browse score was given to young, intermediate and older leaves. The browse score was a 7-point visual estimate of the proportion of leaf biomass removed from each leaf age class defined as follows:

Browse Score	0	1	2	3	4	5	6
% biomass removed	0	1-5	6-25	26-50	51-75	76-95	96-100

Where leaf or stem material had been removed but was uneaten, browse scores were modified so that only the amount eaten was represented by the browse score. Absolute loss in stem height was also measured. As stems were consumed in low amounts, they will not be considered for future discussion in this study.

A simple model relating DM to seedling height for each leaf age class was developed. A sample of seedlings from the low (n = 50), medium (n = 50), and high (n = 25) nutrient treatments were selected across blocks separated into leaf age classes and stem, and then dried at 70 ºC. The model was a linear regression of leaf DM against seedling height for each leaf age class, performed with PROC REG of SAS (SAS Institute Inc. 2003). Prior analysis (PROC GLM, SAS Institute Inc. 2003) showed that nutrient had no significant effect on this relationship.

Leaf intake was estimated as the product of the initial leaf DM and the mid-point range of the proportion of leaf biomass removed, converted from the browse score (as done by McArthur et al. 2000). Estimates of DM intake for each leaf

age class was expressed as the amount of DM eaten per kilogram of body mass for each animal (gDM kg^{-1} BM).

Filming and analysis of feeding pattern

To determine which leaf age class was eaten first by possums in nutrient treatments where young leaves were not offered *ad libitum*, animals were filmed (from 1700 h to 0800 h) on the test night, following the methods of Wiggins et al. (2003). The video was examined to determine what part of each seedling the animal ate first on its initial visit.

Seedlings were scored as having had young foliage browsed first, if the first foliage eaten in the first browsing event on that seedling was young foliage only, or young foliage and less than 50 % of the intermediate leaves. These criteria were used because a bite from the top of a seedling occasionally included some intermediate leaves in addition to young leaves.

Chemical analysis of seedlings

For each test night over six periods of the feeding trial, five seedlings of each treatment from every block were randomly selected, pooled, bagged and stored at –20 ºC for subsequent chemical analysis. These seedlings were then divided into leaf age classes. Chemical components for intermediate and older leaves were replicated five times in all treatments, with the exception of those of formylated phloroglucinol compounds (FPCs), which were replicated three times. Due to limited availability of materials, young leaves were pooled across the five blocks into one sample in the low and medium treatments, and young leaves from blocks 1-3 were pooled and blocks 4 and 5 were also pooled in the high treatment.

Primary chemistry

For nitrogen and plant cell-wall component analysis, freeze dried material was ground though a 1 mm mesh sieve using a cyclotech ® mill. Nitrogen concentration was digested following Lowther (1980). Digested samples were colourimetrically analysed for nitrogen (QuikChem method 13-107-06-2D, Lachat Instruments, Wisconsin, USA) on a continuous flow injection analyser (QuikChem 8000, Lachat instruments). Standard samples of known nitrogen concentration and blank samples were included to validate the efficiency of analyses. Nitrogen results are expressed as % dry matter.

Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (lignin) were determined sequentially following the ANKOM Technology procedures (ANKOM$^{200/220}$ Technology operator's manual 1997).

Secondary chemistry

Essential oils were extracted using dichloromethane with heptadecane as an internal standard (O'Reilly-Wapstra et al. 2004). Two grams of thawed (fresh-frozen) foliage, cut into approximately 1 cm pieces, was soaked in the dichloromethane solvent for 1 h. Extracts were analysed by combined gas chromatography-mass spectrometry (GC-MS), with estimation of both total oils and the main oil component, 1,8-cineole, as described by O'Reilly-Wapstra et al. (2005).

Total phenolics were assayed using the modified Prussian blue assay (Graham 1992). Plant material was prepared and extracted as outlined in Hagerman (1995). The concentration of the total phenolics was determined by using a pure gallic acid standard (Sigma G-7384) and expressed as mg g^{-1} DM equivalents of gallic acid.

Formylated phloroglucinol compounds (FPCs) were extracted as outlined in Wallis et al. (2003) and analysed using high pressure liquid chromatography with diode array UV detection and extraction of chromatograms at 280 nm as described by O'Reilly-Wapstra et al. (2004). Peaks were identified by retention times and UV spectra in comparison to authentic standards and quantified based on the response factors calculated from accurately prepared solutions of sideroxylonal A and sideroxylonal C standards. Representative samples were also analysed by high pressure liquid chromatography combined with negative ion electrospray mass spectrometry to confirm that the molecular weights and tandem MS data of the peaks selected from the UV trace were also consistent with the standards (Eyles et al. 2003). Under these conditions retention times were: sideroxylonal A 11.40 min, sideroxylonal B 15.88 min, and sideroxylonal C 12.02 min. The main FPCs identified were sideroxylonal A, B and C. Sideroxylonals A and C were not baseline resolved and could not be accurately quantified separately, so have been expressed together. As the concentration of sideroxylonal A+C and sideroxylonal B were highly correlated ($r = 0.98$, $P = 0.0001$), and the concentration of essential oils was highly correlated with cineole concentration ($r = 0.99$, $P = 0.0001$), for simplicity, only sideroxylonal A+C and essential oils are described in the results.

Physical analysis of seedlings

For each nutrient treatment and leaf age class, physical leaf characteristics were measured from one leaf from each age class of 15 seedlings. Characteristics of young leaves were measured from a leaf on the node directly below the apical bud. Intermediate and older leaf characteristics were measured from a leaf belonging to the first leaf pair of that section.

Leaf anatomy

Cuticle thickness was measured microscopically on transverse sections approximately 0.05 mm thick, cut approximately half way along the leaves as described in Jordan et al. (2005). Measurements were made on three separate sections from one leaf, for each leaf age class of five plants, for each nutrient treatment.

Leaf biomechanical properties

For logistical reasons, leaf biomechanical properties were measured on leaves collected two months after the feeding trial. However, during this period the nutrient application treatments were continued. The measured biomechanical properties all showed strong correlations with cuticle thickness, the latter measured at the time of the feeding trial ($r > 0.89$, $P < 0.0012$). We therefore considered they were likely to be indicative of the state of the leaves at the time of the trial.

Biomechanical properties were measured within 24 hours of harvest by a punch-and-die test (Aranwela et al. 1999, Sanson et al. 2001) with a Chatillon UTSE-2 force tester (AMETEK Inc., Largo, FL, USA), following Iddles et al. (2003). Four mechanical properties of the leaves were derived from force-displacement curves using Leaf 7a software (M. Logan, Monash University, Vic.); strength, specific strength, work-to-punch, and specific work-to-punch following Read et al. (2003). The trends in strength and specific strength among nutrient treatments and leaf age classes were correlated ($r > 0.85$, $P < 0.004$) to those for work (toughness) and specific work (toughness adjusted for leaf thickness) respectively, therefore only the latter two properties depicting toughness are mentioned.

Calculating intake and chemical and physical characteristics on a whole-seedling basis

Overall intake by pademelons and possums was obtained by summing the intake of each leaf age class within a treatment. Each chemical and physical characteristic was expressed at the whole seedling level using the values from each leaf age class, and weighted for their relative proportions of DM.

Statistical Analyses

All analyses, except correlations, were performed using the general linear model procedure of SAS (PROC GLM) (SAS Institute Inc. 2003)). Residuals were checked for normality and homoscedasticity, and no transformations were necessary. Pair-wise comparisons of treatment least squares means were made using the Tukey-Kramer adjustment for multiple comparisons. The effect of nutrient treatment (low, medium, high) on each chemical and physical seedling characteristic was determined across leaf age classes and at the overall plant level.

Intake (g DM kg^{-1} BM) of leaf age classes, and overall intake of foliage by animals, in relation to each treatment, were analysed for each species according to the models:

$$\textit{Intake of leaf age classes} = \textit{age class} + \textit{treatment} + \textit{carry} + \textit{period} + e$$

$$\textit{Total seedling intake} = \textit{treatment} + \textit{carry} + \textit{period} + \textit{animal} + e$$

where *age class* is the leaf age class (young, intermediate, older), *treatment* is the nutrient treatment (low, medium, high), *carry* is testing for carryover effects from the previous treatment fed to the animal, *period* is the trial feeding period (1-3), and *e* is the residual. All effects were treated as fixed.

We tested the effects of nutrient treatment, animal, carryover and period on the percentage of each leaf age class browsed following the model:

$$\textit{percentage browsed} = \textit{treatment} + \textit{animal} + \textit{age class} + \textit{carry} + \textit{period} + e$$

The correlations between intake and all seedling chemical and physical characteristics by leaf age class (including the ratio of nitrogen to secondary metabolites after Lambdon and Hassall 2005) were tested following Tabachnick

& Fidell (1989), using Pearson's correlation coefficients (PROC CORR, SAS Institute Inc. 2003). Correlations between chemical and/or physical characteristics within leaf age classes across all treatments were also tested using Pearson's correlation coefficient.

Results

Intake of seedlings by pademelons and possums

For each nutrient treatment, pademelons ate significantly more older leaves than young leaves (Figure 1a), and the intake of intermediate leaves ranked between the intake of young and older leaves (Figure 1a). In the low nutrient treatment, intake by possums showed a similar pattern to that of pademelons, with older leaves consumed most, and young leaves the least (Figure 1). In the medium and high nutrient treatments, however, possums ate more of the intermediate leaves than the older leaves (Figure 1b). Young leaves in the medium and high nutrient treatments were eaten least (Figure 1b) but they were not available *ad libitum*. In fact, the video footage showed that in their first visit to individual seedlings, possums browsed young leaves first 90 % (SE = ± 0.03) and 91 % (SE = ± 0.02) of the time in the medium and high nutrient treatments respectively. In contrast, they browsed young leaves first only 37 % (SE = ± 0.10) of the time in the low nutrient treatment. The treatment effect was significant ($F_{2,9} = 27.3$, $P < 0.001$) and young leaves were browsed first significantly more frequently in medium and high nutrient treatments than in the low nutrient treatment.

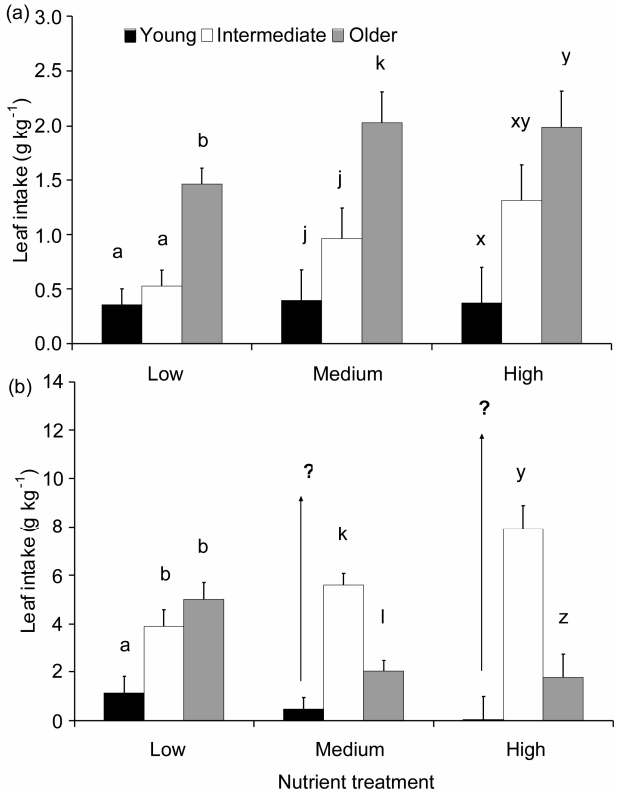

Figure 1. Intake of leaf dry matter per unit animal body mass for leaf age classes by (a) pademelons, and (b) possums, in the three different nutrient treatments. ↑ = Intake not *ad libitum* and consumption for these leaf age classes determined elsewhere. Letters that differ within each nutrient treatment indicate significant differences (α = 0.05 after Tukey-Kramer adjustment for multiple comparisons).

Possums ate much more than pademelons across all nutrient application treatments (up to 10 gDM kgBM^{-1}, compared to pademelons who consumed up to 3.7 gDM kgBM^{-1}). Overall leaf intake by pademelons increased slightly but not significantly with increasing nutrient application, while intake by possumsshowed no such trend.

In the medium and high nutrient treatments young leaves were the most damaged (expressed as percentage browsed) by pademelons, followed by older leaves, then intermediate leaves (Figure 2a). In the low nutrient treatment, the damage on young and older leaves was similar, with intermediate leaves being the least damaged (Figure 2a).

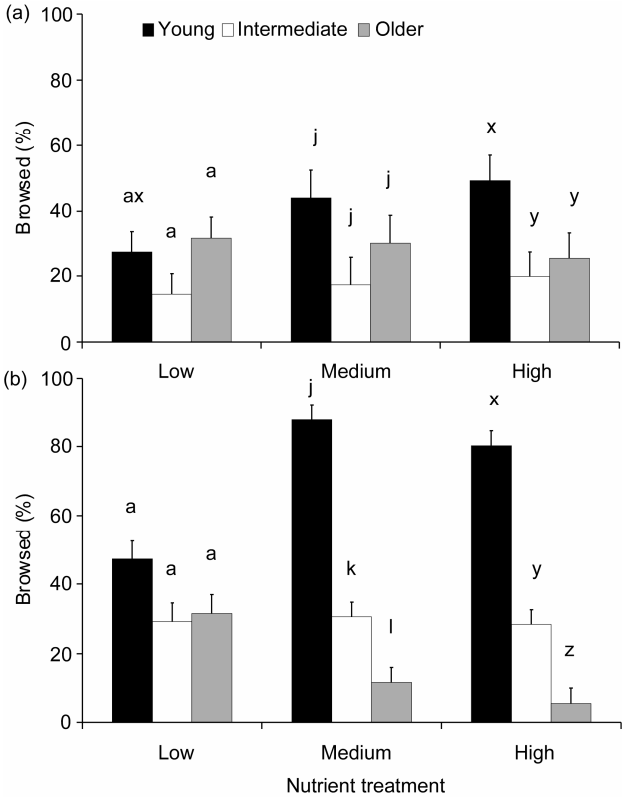

Figure 2. Foliage browsed (expressed as a percentage of what was available) by pademelons (a), and possums (b), in three different nutrient treatments (low, medium, high). Letters that differ within a nutrient treatment indicate significant differences ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons).

Irrespective of treatment, young leaves of seedlings always received the most damage from possums, with the percentage browsed ranging from almost fifty percent in the low nutrient treatment to over eighty percent in the medium and high treatments (Figure 2b). In the medium and high treatments the older leaves were the least damaged, while in the low treatment, older and intermediate leaves incurred approximately the same percentage damage (Figure 2b).

Nutrient effects on seedling height and number of leaves

Seedling height, total number of leaves, and the number of intermediate and older leaves all increased with increasing amounts of nutrients added to the seedlings (Table 1).

Table 1. Seedling characteristics from three different nutrient treatments (low, medium, high). Number of young leaves was always equal to two leaves plus the apical bud. Values are least squares means (±SE). For each variable, values with different superscript letters were significantly different ($\alpha = 0.05$) after Tukey-Kramer adjustment for multiple comparisons.

	Treatment		
Variable	Low	Medium	High
Seedling height (mm)	269 ± 3^a	305 ± 3^b	373 ± 3^c
Total number of leaves	8.2 ± 0.1^a	13.1 ± 0.1^b	15.8 ± 0.1^c
Number of intermediate leaves	3.8 ± 0.1^a	6.3 ± 0.1^b	7.6 ± 0.1^c
Number of older leaves	2.4 ± 0.1^a	4.9 ± 0.1^b	6.2 ± 0.1^c

Within-seedling foliar characteristics

Dry matter, as percentage of fresh mass, increased with leaf age class in the medium and high nutrient treatments, but not in the low nutrient treatment (Table 2). For each leaf age class, foliar nitrogen in the low nutrient treatment was only approximately 30-50 % of that in the medium nutrient treatment and approximately 25-35 % of that in the high nutrient treatment (Table 2). Furthermore, foliar nitrogen decreased with leaf age class (young>intermediate>older) in all three treatments, although this was much less marked in the low nutrient treatment, where the difference between young and intermediate leaves was not significant (Table 2). Neither measure of fibre (NDF and ADF) varied significantly between leaf age classes in the low nutrient treatment, although lignin was significantly higher in young leaves in this treatment (Table 2). In contrast, in the medium and high nutrient treatments, ADF and lignin were significantly lower in the young leaves than other leaf age classes (Table 2).

Seedlings in the low nutrient treatment showed little difference in the amount of total phenolics across leaf age classes (Table 2). In the medium and high nutrient treatments, however, young leaves had much higher phenolic concentrations than intermediate and older leaves (Table 2). In all treatments, young leaves contained much higher concentrations of sideroxylonal A+C and essential oils than intermediate leaves, which in turn were higher than older leaves (Table 2). Sideroxylonal A+C was also highly correlated with essential oils ($n = 9$, $r = 0.97$, $P < 0.0001$).

In the low nutrient treatment, values for work and specific work were significantly lower for intermediate leaves than young and older leaves, while young and older leaves were approximately the same value (Table 2). In contrast, in the medium and high treatments, young leaves required significantly less work and specific work to punch than intermediate and older leaves, while intermediate and older leaves required around the same work to punch (Table 2). In the low nutrient treatment there was no significant difference in cuticle and epidermal thickness between leaf age classes (Table 2). In the medium and high treatments however, young leaves had significantly thinner cuticles than intermediate or older leaves (Table 2)..

Table 2. Primary and secondary leaf chemistry, and physical characteristics for each leaf age class (young, intermediate, older), and at the whole plant level (leaves and stem) of seedlings in the three nutrient treatments (low, medium, high). Values are least squares means ±SE. For detailed description of units, see text. NDF = neutral detergent fibre, ADF = acid detergent fibre. n.d. = not determined. For each variable and plant part, values with different superscript letters within a row were significantly different ($\alpha = 0.05$) after Tukey-Kramer adjustment for multiple comparisons. For each variable for total leaf and stem, values with different letters within a column were significantly different under the same criterion.

			Leaf age class				
Variable	Units	Treatment	Young	Intermediate	Older	Total leaf	Stem
Dry mass	% of fresh mass	Low	44.5 ± 0.5^{a}	47.6 ± 0.5^{b}	44.0 ± 0.5^{a}	45.8 ± 0.3^{a}	42.8 ± 0.3^{x}
		Medium	36.3 ± 0.8^{j}	44.4 ± 0.8^{k}	47.0 ± 0.8^{k}	45.0 ± 0.3^{a}	40.6 ± 0.3^{y}
		High	35.0 ± 0.5^{x}	40.8 ± 0.5^{y}	45.8 ± 0.5^{z}	$42.5 \pm 0.\ 5^{b}$	40.1 ± 0.5^{y}
Nitrogen	% dry mass	Low	0.61 ± 0.03^{a}	0.55 ± 0.01^{a}	0.48 ± 0.01^{b}	0.53 ± 0.03^{a}	0.28 ± 0.03^{x}
		Medium	1.88 ± 0.10^{j}	1.06 ± 0.04^{k}	0.87 ± 0.04^{l}	1.02 ± 0.03^{b}	0.54 ± 0.03^{y}
		High	2.31 ± 0.08^{x}	1.54 ± 0.05^{y}	1.21 ± 0.05^{z}	1.43 ± 0.03^{c}	0.69 ± 0.03^{z}
NDF	% dry mass	Low	17.7 ± 1.0^{a}	16.3 ± 0.4^{a}	16.2 ± 0.4^{a}	16.4 ± 0.6^{a}	48.1 ± 1.1^{x}
		Medium	16.5 ± 1.2^{jk}	16.0 ± 0.5^{j}	19.3 ± 0.5^{k}	17.9 ± 0.6^{a}	48.8 ± 1.1^{x}
		High	15.0 ± 1.4^{x}	18.3 ± 0.9^{x}	18.7 ± 0.9^{x}	18.3 ± 0.6^{a}	51.6 ± 1.2^{x}
ADF	% dry mass	Low	10.9 ± 0.5^{a}	10.9 ± 0.2^{a}	10.8 ± 0.2^{a}	10.9 ± 0.3^{a}	34.4 ± 0.8^{x}
		Medium	7.7 ± 0.5^{j}	10.4 ± 0.2^{k}	12.1 ± 0.2^{l}	11.1 ± 0.3^{a}	34.5 ± 0.8^{x}
		High	6.8 ± 0.4^{x}	10.21 ± 0.3^{y}	11.67 ± 0.3^{z}	10.7 ± 0.3^{a}	37.0 ± 0.5^{x}
Lignin	% dry mass	Low	4.44 ± 0.42^{a}	3.83 ± 0.19^{ab}	3.26 ± 0.19^{b}	3.68 ± 0.16^{a}	7.6 ± 0.30^{x}
		Medium	2.29 ± 0.29^{j}	4.04 ± 0.13^{k}	4.03 ± 0.13^{k}	3.91 ± 0.16^{a}	6.78 ± 0.30^{x}
		High	1.68 ± 0.28^{x}	3.95 ± 0.18^{y}	3.93 ± 0.18^{y}	3.81 ± 0.16^{a}	7.49 ± 0.30^{x}
Total phenolics	mg g^{-1} dry mass	Low	132 ± 17^{a}	123 ± 8^{a}	124 ± 8^{a}	124 ± 4^{a}	79 ± 5^{x}
	gallic acid equivalents	Medium	135 ± 14^{j}	105 ± 9^{k}	94 ± 9^{k}	104 ± 4^{b}	59 ± 5^{y}
		High	161 ± 10^{x}	99 ± 6^{y}	110 ± 6^{y}	105 ± 4^{b}	48 ± 5^{xy}

- continued

Table 2 (continued).

Variable	Units		Leaf age class Young	Intermediate	Older	Total leaf	Stem
Sideroxylonal A+C	mg g^{-1} dry mass	Low	3.45 ± 0.18^{a}	1.86 ± 0.10^{b}	0.87 ± 0.10^{c}	1.66 ± 0.09^{a}	1.20 ± 0.18^{x}
		Medium	4.64 ± 0.19^{j}	2.60 ± 0.11^{k}	1.35 ± 0.11^{l}	2.06 ± 0.09^{b}	1.20 ± 0.18^{x}
		High	3.49 ± 0.17^{x}	2.51 ± 0.14^{y}	1.58 ± 0.14^{z}	2.14 ± 0.09^{b}	1.14 ± 0.22^{x}
Essential oils	mg g^{-1} dry mass	Low	1.30 ± 0.09^{a}	0.30 ± 0.04^{b}	0.20 ± 0.04^{b}	0.38 ± 0.08^{a}	0.10 ± 0.03^{x}
	heptadecane equivalents	Medium	2.58 ± 0.23^{j}	0.88 ± 0.14^{k}	0.32 ± 0.14^{k}	0.70 ± 0.08^{b}	0.18 ± 0.03^{x}
		High	1.88 ± 0.21^{x}	1.30 ± 0.13^{x}	0.30 ± 0.13^{y}	0.87 ± 0.08^{b}	0.14 ± 0.03^{x}
Work	J m^{-2}	Low	0.93 ± 0.13^{a}	1.65 ± 0.13^{b}	1.11 ± 0.03^{a}	1.36 ± 0.12^{a}	n.d.
		Medium	0.26 ± 0.09^{j}	1.31 ± 0.09^{k}	1.27 ± 0.09^{k}	1.35 ± 0.12^{a}	n.d.
		High	0.25 ± 0.13^{x}	1.28 ± 0.13^{y}	1.49 ± 0.13^{y}	1.32 ± 0.12^{a}	n.d.
Specific work	J m^{-2} m^{-1}	Low	3.67 ± 0.46^{a}	5.53 ± 0.46^{b}	3.91 ± 0.46^{a}	4.70 ± 0.38^{a}	n.d.
		Medium	1.63 ± 0.28^{j}	4.43 ± 0.28^{k}	4.11 ± 0.28^{k}	4.51 ± 0.38^{a}	n.d.
		High	1.52 ± 0.43^{x}	4.27 ± 0.43^{y}	5.02 ± 0.43^{y}	4.46 ± 0.38^{a}	n.d.
Cuticle thickness	µm	Low	6.23 ± 0.71^{a}	8.88 ± 0.71^{a}	7.28 ± 0.71^{a}	7.96 ± 0.41^{a}	n.d.
		Medium	2.80 ± 0.36^{j}	7.54 ± 0.36^{k}	6.31 ± 0.36^{k}	6.51 ± 0.41^{a}	n.d.
		High	2.85 ± 0.27^{x}	6.73 ± 0.27^{y}	7.65 ± 0.27^{y}	6.94 ± 0.41^{a}	n.d.

Whole seedling characteristics
There was a significant nutrient effect on all primary and secondary leaf characteristics measured, except cell wall components and cuticle thickness (Table 2). Mechanical properties of the leaves varied little at the whole seedling level, except that leaves in the high nutrient treatment required less strength to punch than those of medium and low treatments (Table 2).

Intake of leaves in relation to plant characteristics
Leaf intake by pademelons decreased significantly with sideroxylonal A+C concentration across all leaf age classes and nutrient treatments ($n = 9$, $r = -0.77$, $P < 0.05$, Figure 3a & 3b). Essential oils showed a similar trend ($n = 9$, $r = -0.66$, $P = 0.052$, Figure 3b). No other chemical or physical factors correlated significantly with intake by pademelons. In contrast, no single chemical or physical factor was correlated consistently with intake of leaves by possums across all leaf age classes and nutrient treatments.

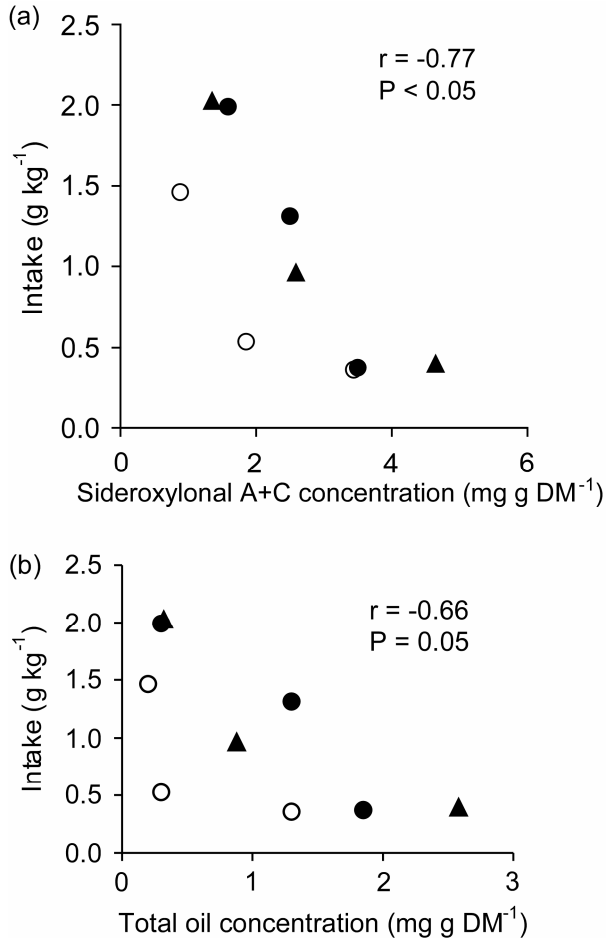

Figure 3. Relationship between mean intake of leaf dry matter per unit animal body mass by pademelons and (a) sideroxylonal A+C concentration, and (b) essential oil concentration, expressed as milligrams per gram dry matter. Essential oil concentration is measured as heptadecane equivalents. Each of the nutrient application treatments is given a different symbol where *open circles* = low, *closed triangles* = medium, and *closed circles* = high.

The relationship of leaf intake by pademelons to the ratio of nitrogen to sideroxylonal A+C concentration reduced the strength of the correlation between intake and sideroxylonal A+C ($n = 9$, $r = -0.60$, $P > 0.05$). The ratio of nitrogen to essential oil concentration was significantly correlated to leaf intake by pademelons ($n=9$, $r= -0.70$, $P < 0.05$) and this was a slight improvement over the simple

correlation between intake and essential oils. Neither the ratio of nitrogen to sideroxylonal A+C concentration, nor the ratio of nitrogen to essential oil concentration correlated significantly with leaf intake by possums.

Discussion

Intake of seedlings by pademelons and possums

Overall, possums and pademelons generally ate different amounts from different leaf age classes of *E. nitens*, and the relative use of these leaf classes by possums was altered by the nutrient treatment applied to seedlings. Pademelons, irrespective of nutrient treatment, always ate more older leaves than young leaves. Similarly possums ate more older and intermediate leaves than young leaves in the low nutrient treatment, but their intake of young leaves in the medium and high nutrient treatments is likely to be under-represented as young leaves were not available *ad libitum*. Video evidence showed that possums ate young leaves first over other leaf age classes in the medium and high nutrient treatments, suggesting their relative use would have been greater given the opportunity.

Whole seeding vs. within-seedling foliar characteristics

Consistent with Close et al. (2005) leaf age classes differed in important chemical components. Our study showed that leaf age classes also varied in physical properties: toughness and cuticle thickness.

Contrary to the expectations of Lambdon and Hassall (2005), who suggested that the chemical composition of leaves of different ages is usually less variable than leaves between individual plants, the differences in all of the chemical and physical characteristics between leaf age classes within a nutrient regime were large, and with the exception of nitrogen content, were greater than the overall differences between nutrient treatments. For example, the concentration of sideroxylonal A+C varied by as much as 400 % between leaf age classes, but varied by only about 100% between nutrient treatments. As noted by Close et al. (2005), the higher variation exhibited by leaf age classes demonstrates that whole-plant analyses do not provide complete information about within-plant levels of some constituents. As our large differences amongst leaf age classes were not constant across nutrient treatments, this implies that within plant variation may be very significant for understanding selection processes occurring during browsing.

Intake of leaves in relation to plant characteristics

The concentration of sideroxylonals and/or essential oil compounds in the leaves appear to be the major determinants of herbivory by pademelons because of the inverse relationship between the concentrations of these chemicals and the pademelons' relative use of leaf age classes. We cannot distinguish the effects of these components on intake because they are strongly correlated in eucalypts (e.g. O'Reilly-Wapstra et al. 2004, Lawler et al. 2000 and this study). Lawler et al. (1999) were able to separate effects of essential oils and FPCs by using pure compounds to assess intake by herbivores, and later suggested that folivorous marsupials may use the concentration of essential oils (as assessed by smell) to estimate the concentration of FPCs (which are not volatile) before consumption (Lawler et al. 2000). Interestingly, the leaves selected by pademelons were those lowest in nitrogen, which suggests that the deterrent produced by the secondary compounds outweighed the attraction of foliage high in nitrogen. The ratio of nitrogen to sideroxylonal A+C concentration did not explain intake as well as sideroxylonal A+C did in isolation, which argues against Lambdon and Hassall's (2005) proposal that the ratio of nutrients to secondary chemicals may be more important in determining an animal's intake than either component in isolation.

This is the first study to show a direct association between feeding by pademelons and sideroxylonal concentration. In a previous study (Lawler and Foley 1999) intake of different types of *E. ovata*

foliage by pademelons could not be associated with any chemical characteristic, even though intake by pademelons was correlated strongly with that of ringtail possums, which was in turn correlated to sideroxylonal and essential oil concentration. Sideroxylonals can be deterrents for ringtail possums (Lawler et al. 2000) and brushtail possums (O'Reilly-Wapstra et al. 2004). However, other studies have shown that condensed tannins may be important in influencing intake of eucalypt foliage by brushtail possums (Marsh et al. 2003a, 2003b, O'Reilly Wapstra et al. 2005). As there are no condensed tannins in *E. nitens* (Close et al. 2005), this is not relevant to our study.

Intake of leaf age classes by possums across the three nutrient treatments could not be explained consistently by a single chemical or physical characteristic. In our medium and high nutrient treatments, the video footage indicated that possums preferentially consumed young leaves in spite of high levels of sideroxylonals and essential oils. These young leaves did, however, have the highest nitrogen concentration of any of the leaf age classes. Thus, either these secondary compounds provide little or no deterrence to possums, or any deterrence is outweighed by other factors, such as high nitrogen. The greater relative use of older leaves by possums in the low nutrient treatment, where foliar nitrogen content was very low and relatively constant among leaf age classes, favours the latter explanation, because intake was highest for older leaves and lowest for young leaves in that treatment, inversely matching the pattern in essential oils and sideroxylonal A+C. Thus, if high nitrogen levels allow possums to offset the costs of eating young leaves high in essential oils and sideroxylonals, we suggest that these secondary compounds may be more important at low foliar nitrogen levels. Similar interactions between nutrient levels with deterrent effects of plant secondary compounds have been demonstrated before for insect herbivores (Behmer et al. 2002), and mammalian herbivores (Wang and Provenza 1997).

Although we found significant differences in physical characteristics between leaf age classes, they were not negatively associated with intake, which therefore suggests that toughness or fibre content of these leaves was not adequate to act as a deterrent to pademelon and possum herbivory.

Caution should be made when comparing differences in chemical and physical characteristics between leaf age classes under different nutrient treatments. For example the low nutrient treatment grew fewer leaf pairs (Table 1), and young leaves of this treatment may have been older than young leaves of the high nutrient treatment. This could explain some of the differences in physical and chemical characteristics of leaf age classes between nutrient treatments. For example, young leaves in the low nutrient treatment had very high values for lignin content and cuticle thickness, in comparison to the other nutrient treatments. However, differences in age of the same leaf age classes across nutrient treatments cannot always explain the observed patterns in the animals' relative use of the leaves, as pademelons showed the same intake patterns across all nutrient treatments. Although intake by possums showed different patterns across nutrient treatments, we would expect a quantitative effect on intake if differences in leaf age caused these differences (i.e. in the low nutrient treatment, intake of young leaves could resemble that of intermediate leaves from the other nutrient treatments), but overall trends with age should be similar across treatments. However, possum relative use of foliage increased with the age of the leaves for the low nutrient but decreased with leaf age in the medium and high nutrient treatments.

Ecological and evolutionary implications

Plant defence theories suggest that the most vulnerable or valuable plant parts should be heavily defended against herbivory (McKey 1979, Bryant et al. 1991, Zangeral and Bazzaz 1992). Young developing leaves are vulnerable to herbivore attack because they cannot be sclerified before they are completely expanded (Coley 1983, Choong 1996) and often contain the most nutrients and water

(Raupp and Denno 1983, Nichols-Orians and Shultz 1990). Also, the large investment into these organs with as yet, relatively little or no return, makes them valuable to the plant (Harper 1989). Therefore, it has been suggested that chemical defenses should be most concentrated in the youngest leaves (Laitinen et al. 2002).

The distribution of key deterrent chemical components, sideroxylonals and essential oils, and the patterns of herbivory by pademelons found in the present study are consistent with such theories. However, possums ate the most valuable leaves of seedlings first (i.e. young leaves), in the medium and high nutrient treatments.

Rousi et al. (1993) found comparable differences in the intake of birch seedlings by voles and hares and speculated that such variability between different herbivores is difficult to reconcile with generalised hypotheses on plant resistance to herbivory. Alternatively, plants may defend themselves from the kind of herbivory that had been the biggest threat over evolutionary time. In our system, this would suggest either that pademelons or similar herbivores have had a stronger influence on the development of defence chemicals, or that possums have adapted to tolerate the defence chemicals present in *E. nitens* seedlings.

Although there is much in the literature regarding the value of plant parts, there is little documenting how selective herbivory of plant parts affects plant fitness. The greatest effect of herbivory on plant survival is likely to be at the seedling stage (Maron 1997, Watkinson 1997) and selective predation of preferred seedling species by herbivores has far-reaching consequences for plant community development (Hanley 1998). Selective intake of particular plant parts, not just selective intake between plants, may also affect seedling fitness and plant communities.

Repeated browsing of the upper foliage is likely to affect seedling survival and long term tree form. Although two simulated browsing studies on eucalypt seedlings showed little effect on seedling growth or survival by removal of the upper leaves (Nielsen and Pataczek 1991, Wilkinson and Nielsen 1995) the degree to which these results can be extrapolated to the field may be limited, because foliage was removed on only one or two occasions. In contrast, herbivory can promote the development of multiple leaders in eucalypts (Bulinski and McArthur 1999) and repeated damage to the growing tips of eucalypt seedlings, a pattern demonstrated by possums in two of three nutrient treatments in our study, can cause tree malformation (Volker and Orme 1988). We therefore suggest that selective browsing of older leaves from seedlings, as shown by pademelons, may have less impact on plant fitness than selective browsing of young leaves by possums for two reasons. First, it is unlikely to alter the growth form of the seedlings, and second, older leaves have less photosynthetic potential than younger leaves.

It is possible, however, that the impact of herbivores on seedling fitness does not necessarily directly reflect the relative use of, or preference for, a particular plant part, because plant parts are not always present in equal biomass. Although pademelons ate more older leaves than medium or young leaves in all nutrient treatments, they did as much damage to the young leaves, in terms of percent of available foliage browsed, as the older leaves (Figure 2a). Therefore the damage caused by pademelons on plant fitness may, in fact, be greater than suggested by intake measures alone. Our results suggest that possums may have a strong effect on plant fitness on fertile soils because they not only ate valuable young foliage first, but they caused more damage to the young foliage (Figure 2b). Thus, differential herbivory, resulting from the variation in key chemical constituents (notably nitrogen, sideroxylonals and essential oils) among plant parts and in response to soil nutrient levels, is likely to affect plant fitness significantly.

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